Raman Detection of Protein Interfacial Conformations

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Abstract: The surface adsorbed protein conformations onto the vaccine adjuvants were observed with a Raman spectroscopy by using the maximum adsorption conditions described previously. The adsorbed state Raman vibrational spectra and subsequent spectral analysis display no conformational changes for BSA or IgG relative to their native species in solution.
Key words: adsorption, protein conformation, raman spectroscopy, vaccine adjuvants

Protein adsorption and interfacial behavior are encountered in many biological, medical and industrial applications such as membrane receptor binding, protein chromatography, solid-phase immunoassays, vaccine adjuvants, drug delivery devices and medical implants. In some applications like vaccine adjuvants, adsorption plays an important functional role, but in other cases it is an unwanted side effect. To prevent losses or activity modifications due to adsorption and to further optimize functional surface interactions, a more complete understanding of the mechanism of protein adsorption to solid surfaces is necessary. The potential mechanisms of protein-surface interactions are chemical forces such as electrostatic interactions, hydrogen bonding, hydrophobic interactions, van der Waals and charge-transfer interactions that are responsible for maintaining native protein conformation (Andrade, 1985; Horbett, 1987).

Orientations and structures of adsorbed proteins on a solid surface have been probed with a variety of techniques, including circular dichroism (CD) (Norde *et al.*, 1986), attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) (Giroux and Copper, 1990; Lu and Park, 1991), and total internal reflectance fluorescence spectroscopy (TIRF) (Shibata and Lenhoff, 1992). However, there have been no previous studies on the structural conformations of polypeptides adsorbed to mineral vaccine adjuvants.

In this study, we have used Raman spectroscopy to probe the surface sensitivity of two native proteins, bovine serum albumin (BSA) and immunoglobulin G (IgG), to adsorption onto both aluminum oxyhydroxide (Alhydrogel) and aluminum phosphate gels (Adju-Phos). Commercially available Alhydrogel and aluminum phosphate gel vaccine adjuvants were chosen as hydrophilic colloidal surfaces. These high surface area gels adsorb significant amounts of protein which results in high surface concentrations; this enables normal Raman spectroscopy to be an effective technique for the analysis of surface-bound protein conformations. Raman spectroscopy is a powerful method for the study of surface-adsorbed biomolecules because water has a weak scattering cross-section that does not mask the vibrations of interest. Furthermore, because of the light scattering nature of the Raman technique, no special requirements are necessary to analyze solid samples. Also, there is no interference from the vaccine adjuvants because the major Alhydrogel and Adju-Phos vibrations do not overlap with the polypeptide backbone vibrations (Hem and White, 1984). The Amide I and III Raman vibrations of polypeptides and proteins are known to have distinct band positions for α-helix, β-sheet and disordered protein structure (Brynda, *et al.*, 1990; Fukumaura and Hayashi, 1990). To quantitate the observed conformational changes, a Raman Spectral Analysis Program (RSAP) is used in this work (Przybycien and Bailey, 1989). RSAP compares the band position of the amide I band of the protein under investigation with a library of known Raman amide I bands for a variety of secondary structures; therefore we can quantitate changes in conformational structure.

The objective of this work is to determine the surface conformation of the adsorbed state of native proteins to the colloidal polymer systems used in vaccine adjuvants by vibrational spectroscopy.

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Materials and Methods

Materials

The mineral adjuvants, aluminum oxyhydroxide (Alhydrogel) and aluminum phosphate gel (Adju-Phos) were commercially obtained from Seargnt (Clifton, NJ, USA). All other chemicals and proteins were obtained from Sigma (St. Louis, MO, USA).

Raman spectroscopy

Previously determined maximum adsorption conditions were used to obtain the samples of adsorbed forms of BSA and IgG in contact with the adjuvants, aluminum oxyhydroxide and aluminum phosphate gels.

Samples for Raman spectroscopy were sealed either between two microscope slides with an O-ring or in cylindrical tubes and maintained at 4°C by a stream of cold air. All Raman spectra were taken with a Spex 1403; 0.85 m double monochromator equipped with photon counting electronics, a cooled Hammamatsu R 928 multi-alkali photomultiplier detector, interfaced with an IBM computer for operation and analysis. The laser excitation source was a Coherent Ar^+ (Innova 90-5) laser; typical laser power was 150~200 mW. Benzene was used for frequency calibration. The spectral resolution was 5 cm^{-1}. The scattered radiation was observed at 90° to the incident beam.

To quantitate the observed structural changes, the spectral differences were enhanced by a data subtraction and curve-fitting program similar to that of Williams (1983). The RSAP program is operated by comparing an unknown protein structure to a large data base of Raman vibrational bands of known protein structures (Przybycien and Bailey, 1989). Based on our experience with the application of RSAP to BSA and IgG in solution and in adsorbed forms, the secondary structure composition obtained is reproducible to within 3%.

Results and Discussion

Raman spectra and spectral analysis of adsorbed species

The maximum adsorption pH and concentration conditions established previously (Jang *et al.*, 1997) were used to prepare adjuvant-adsorbed BSA and IgG samples for Raman spectroscopy. The adsorbed forms of BSA and IgG in contact with the adjuvants, aluminum oxyhydroxide and aluminum phosphate gels, have been investigated by Raman spectroscopy. The Raman spectra of the adsorbed state of BSA to aluminum-containing adjuvants are shown in Fig. 1. The Raman vibrational spectrum of BSA (Fig. 1a) in aqueous solution displays

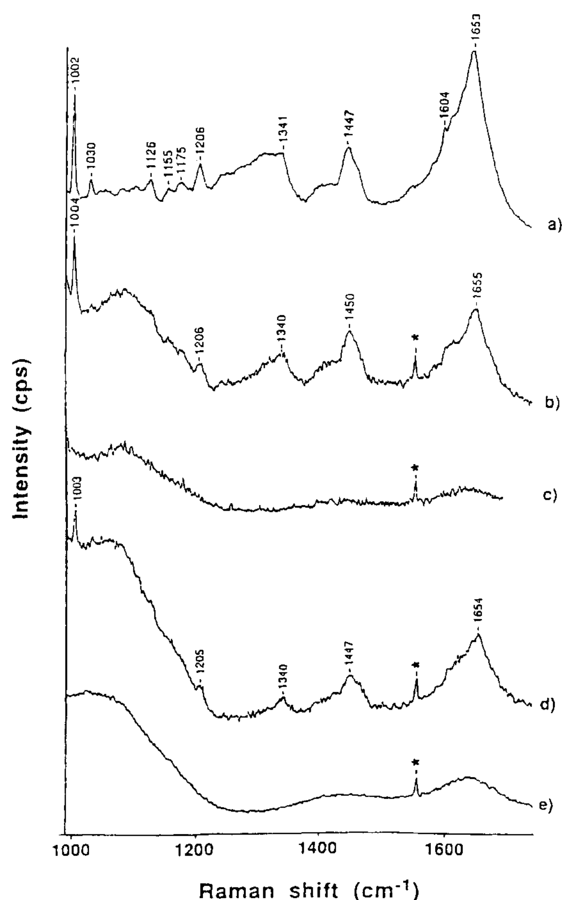

Fig. 1. Raman spectra of: a) BSA (50 mg/ml) in 150 mM NaCl, pH 6.2; b) adsorbed BSA-Alhydrogel; c) Alhydrogel; d) adsorbed BSA-aluminum phosphate gel; e) aluminum phosphate gel. Experiment conditions: laser power 200 mW, 488 nm excitation; integration time 1 cm^{-1}/2 sec; resolution 5 cm^{-1}. Raman bands marked with asterisks are attributable to the sample holder.

the characteristic amide I (1653 cm^{-1}) band indicative of a secondary structure with high α-helical content. The amide III band is indistinguishable in the BSA spectrum. The strong phenylalanine band is observed at 1002 cm^{-1} (Thomas *et al.*, 1990). Other Raman frequencies of BSA are summarized in Table I. The spectrum of Alhydrogel (Fig. 1c) displays a broad band centered at ~1100 cm^{-1} and a broad H-O-H bending vibration at ~1650 cm^{-1}. The aluminum oxyhydroxide adsorbed spectrum of BSA is also reproduced in Fig. 1b). The Alhydrogel bands are unmodified in Fig. 1b) relative to Fig. 1c). The most obvious difference in the adsorbed BSA spectra relative to BSA in solution is the loss of intensity in the 1650 cm^{-1} region. This is attributed to a decreased H-O-H bending vibration because less water is present in the BSA-adjuvant adsorbed pellet. In Fig. 1b) there is a slight upshift of the phenylalanine ring-breathing mode at 1004 cm^{-1}, the aliphatic side chain CH_2 deformation mode at 1341 and

Table 1. Raman frequencies and assignments of BSA and IgG in solution[a]

Frequency (cm^{-1})		Assignment
BSA	IgG	
1002	1004	Phe ring
	1013	Trp ring
1030	1031	Phe ring
1126	1127	C-C stretch
1155, 1175		CH_3 rock
1206	1210	Tyr ring
	1237	Amide III
1341	1339	CH_2 deformation
	1360	Tyr ring
1447	1449	CH_2 deformation
	1460	CH_3 deformation
	1556	Trp ring
1604		Phe ring
1618	1619	Tyr ring
1653		Amide I (α-helix)+H_2O
	1668	Amide I (β-sheet)+H_2O

[a]Raman frequency assignments from Thomas *et al.* (1990).

1450 cm^{-1} and the amide I mode at 1655 cm^{-1}. A similar Raman spectrum is observed for BSA upon adsorption to Adju-Phos with several small bandshifts at 1003 cm^{-1}, 1205 cm^{-1} and 1654 cm^{-1} (Fig. 1d). The background of the adsorbed state of BSA to Adju-Phos is from adjuvant bands (Fig. 1e).

Both of the solution and adsorbed state Raman spectra of IgG (Fig. 2) indicate a high β-sheet structure owing to the amide I and III bands at ~1670 and 1240 cm^{-1}. Raman frequencies of IgG in solution are summarized in Table I. The bandshifts of IgG observed in going from solution to an adsorbed form display similar trends to those observed for BSA. A similar Raman spectrum has also been obtained for IgG adsorbed to aluminum phosphate adjuvant.

These small reproducible vibrational shifts have been investigated with a spectral analysis computer program (RSAP) that fits the peaks to known secondary structures and detects changes in protein conformation. This type of empirical analysis has been used successfully previously to interpret the vibrational data of Raman and FTIR spectra (Susi *et al.*, 1985; Williams, 1986; Przybycien and Bailey, 1989).

Table II summarizes the RSAP results for BSA and IgG as a solid, in solution, and adsorbed to Alhydrogel and Adju-Phos. The program confirms the high α-helical content of BSA and the equally high β-sheet content of IgG. The data for each of the sample types in Table II represents an average of at least three data set analyses; the standard deviations are included except for the data relating to the Adju-Phos surface. For both

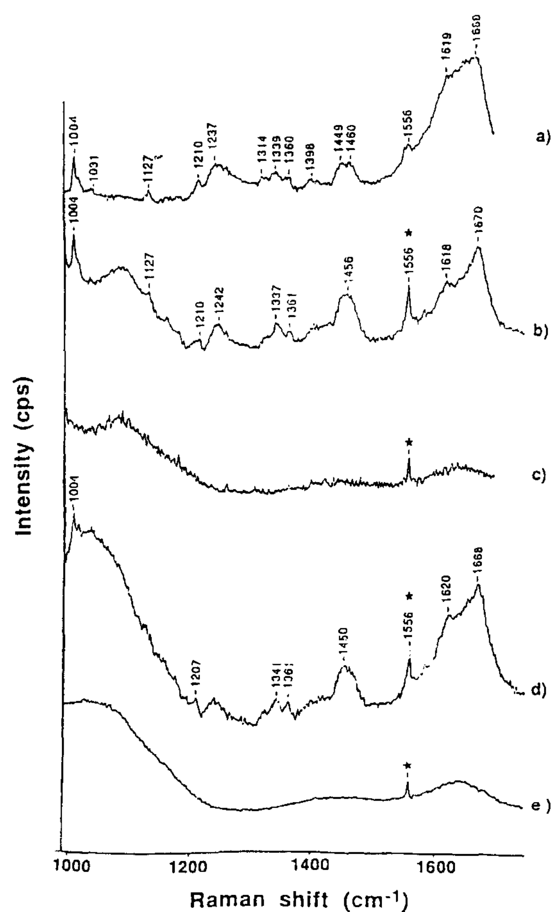

Fig. 2. Raman spectra of: a) IgG (40 mg/ml) in 150 mM NaCl, pH 6.0; b) adsorbed IgG-Alhydrogel; c) Alhydrogel; d) adsorbed IgG-aluminum phosphate gel; e) aluminum phosphate gel. Experiment conditions: laser power 150 mW, 488 nm excitation; integration time 1 cm^{-1}/2 sec; resolution 5 cm^{-1}. Raman bands marked with asterisks are attributable to the sample holder.

BSA and IgG there are observable structural changes between their solid and solution forms. In going from the solid to the liquid form there is a slight decrease in β-sheet and a slight increase in α-helical and random coil content. Similar trends have been observed previously (Thomas *et al.*, 1990), and have been suggested to arise from self-aggregation of the protein molecules. Of major concern in this study is the question of structural differences between the adsorbed and non-adsorbed forms for both BSA and IgG. For this reason, the solution state of the protein is chosen as a comparison state. Furthermore, because the wet form of the antigen-adjuvant pellet samples is used to take the Raman spectrum, the high frequency Raman spectrum of the adsorbed state protein shows intense O-H stretching around 3300 cm^{-1} which indicates the presence of water. Therefore, the solution non-adsorbed state is the best reference point for the adsorbed states of the proteins, in-

Table 2. Analysis of protein secondary structures for different forms of BSA & IgG

Sample	Type	Types of secondary structure		
		α-helix	β-sheet	Random coil
BSA	solid	0.65±0.01	0.17±0.01	0.18±0.01
	solution	0.71±0.03	0.09±0.01	0.20±0.03
	adsorbed[a]	0.70±0.02	0.09±0.02	0.21±0.02
	adsorbed[b]	0.70±0.01	0.09±0.01	0.21±0.01
IgG	solid	0.09±0.01	0.61±0.01	0.30±0.00
	solution	0.05±0.01	0.71±0.03	0.24±0.03
	adsorbed[a]	0.04±0.01	0.73±0.02	0.23±0.02
	adsorbed[b]	0.03±0.01	0.71±0.01	0.26±0.01

[a]adsorbed to the Alhydrogel surface
[b]adsorbed to the Adju-Phos surface

stead of the solid state. Table II shows that the contents of the α-helix, β-sheet and random coil structures are very close to each other for both non-adsorbed and adsorbed states of BSA. For IgG, the composition of protein secondary structures is very slightly different. Examination of the error limits and the spectral analysis results for the solution state and adsorbed forms of BSA and IgG indicates that there is no statistical difference in conformation between these two physical states. This leads us to one of the principal conclusions of this work: that the two stable, globular proteins under investigation, BSA and IgG, do not undergo any conformational changes upon adsorption to the vaccine adjuvants, Alhydrogel and aluminum phosphate gel. In one series of Raman experiments, this constant secondary structure conformation was still maintained after nine days (data not shown). No additional time points were obtained. The adsorbed forms of BSA and IgG do not display even the slightly modified conformations of the solid forms of these proteins. This indicates that negligible protein-protein interactions are induced by adsorption to Alhydrogel and Adju-Phos.

This study was the first observation of protein structure adsorbed to vaccine adjuvant surfaces by Raman spectroscopy. No structural changes between the solution and surface forms of the proteins were observed. This is not an unexpected result because only a small environmental perturbation is anticipated for the adsorption of globular proteins to hydrophilic adjuvant surfaces. For vaccine development work, this is a promising result; however, at present we cannot conclude that this is a universal result. For this reason, a study has undertaken to examine several less stable, hydrophobic proteins that may display surface-induced conformational changes.

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